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Preliminary observations of the gustatory function of sensilla on antennae of the female *Spodoptera littoralis* (Lepidoptera: Noctuidae): from single compounds to complex natural stimuli.

Running Title: gustatory function of sensilla on antennae of the female Spodoptera littoralis

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Abstract

The gustatory system in insects is composed of detectors screening different panels of ligands, which enable or suppress life behaviors depending on the context. Single sensillum electrophysiology recordings were conducted from the antennal sensilla chaetica of an adult female moth of *Spodoptera littoralis* to ascertain whether these sensilla have a gustatory function. Five test stimuli (NaCl, sucrose, ethanol, green cotton leaves extract, and conspecific larval frass extract) were used to stimulate each sensillum. Two types of gustatory sensilla chaetica located on the same flagellomere of the distal third of the antenna were characterized, each sensillum enclosed three gustatory receptor neurons (N1, N2, and N3). Overall, responses (spikes/s) were higher in the case of low concentration of sucrose and higher concentration of ethanol than of salt and higher concentration of sucrose. Individual differences were observed in the response patterns of these sensilla to the tested stimuli but functional sensillum types could not be identified. Higher concentrations of sucrose, ethanol extracts of green cotton leaves, or conspecific larval frass significantly stimulated the same neuron of ventral and lateral sensilla chaetica. Response patterns revealed that antennal gustatory sensilla contain gustatory neurons, which are possible receptors for host-plant recognition. Moreover, stimulation of the female's antennae with phagostimulative sucrose evoked activation of the proboscis extension reflex with dose-dependent responses. Differences in sensilla distribution and their response patterns suggest that gustatory sensilla on the antennae of the female *S. littoralis* have a key role in adaptation and host plant recognition.

Keywords: Moths, phagostimulant, Extracellular recording, Proboscis extension reflex (PER).

Introduction

Egypt, along with other Mediterranean and Middle East, is plagued by the cotton leafworm, *Spodoptera littoralis* (Boisd.), a destructive pest with approximately 112 hosts from diverse families (1-3).

Chemical communication is a critical guide for insects to engage in behaviors such as mating, host-finding, and oviposition. Moths are drawn to host plants by odorant mixes from a distance, but the final choice to begin feeding or laying eggs requires taste (4-6). The female *S. littoralis* deposits her eggs on

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the underside of the leaves of the host plant in clusters. According to early theoretical research, female moths prefer to oviposit on plants where their progeny will do better, forming a fixed hierarchy that reflects the suitability of the various host plant species for larval development (7-9).

The balance of phagostimulants and phagodeterrents influences taste, which is important in choosing an adequate food source (10-12). The chemoreceptors' sensitivity to deterrents plays a more significant role in determining hosts range than does the insect's adaptation to specific phagostimulants (13).

Taste stimuli might be simple compounds or multimolecular combinations (14). Gustatory sensilla can detect non-volatile compounds such as plant metabolites in the wax layer or molecules known to seep into the surface of leaves, in addition to token stimuli such as secondary plant components (12.15-16). Gustatory sensilla are found on appendages such as antennae, proboscis, maxillary and labial palpi, legs, and the ovipositor in insects (5.6.17-19). Gustatory sensory neurons (GSNs) are found within gustatory sensilla that act as a link between insects and their chemical environment (20).

Nonvolatile compounds such as sugars, bitters, amino acids, and secondary plant metabolites can be detected by GSNs (21). Chemical cues from insects or plants can inform females of a wide range of phytophagous insects whether the host plant is suitable for oviposition or is already occupied by their conspecifics (22-23). An earlier study revealed that the female *S. littoralis* avoided laying eggs on likely host plants where their conspecific larvae were already present due to a shortage of food or space for their new offspring (23).

Six sensilla chaetica were identified in fixed locations on each flagellomere of the antennal flagellum of the female *S. littoralis*, except for the last distal segment, which had a higher multiplication of them (18). Sensilla chaetica are long, hair-like structures that are different from other similar sensilla by having thicker walls (5.24-25). In sensilla chaetica, the presence of a terminal pore is usually associated with gustatory function and a flexible basal socket with tactile function (25). The five canonical taste qualities to which insects respond are sweet, bitter, sour, salty, and umami. However, not all the tastes that insects are attracted to fall into this category. Insect tastant classifications can be based on chemical structure, whether the reaction is appetitive or deterrent (26). According to Reiter et al. (27), every tastant may elicit a different spatiotemporal neural representation. Each taste sensillum contains one or more bipolar neurons and the dendrites are protected by extensions of the cuticle (25.28). Tastants can enter the lymph that envelop the dendrites through a pore at the tip of the sensillum's shaft. A train of action potentials is produced by the neuron when the tastant activates the appropriate protein receptor(s) on the dendrite (26).

Previous studies have described the electrophysiological characteristics of the female *S. littoralis* antennal gustatory sensilla (29-31), but it is unknown how they react to complex ligands of either host or non-host origin. Popescu et al., (31) found that female *S. littoralis* antennal gustatory sensilla were activated by sugars or NaCl, with identical response patterns across the whole flagellum of the antenna.

Primary plant metabolites include sugars, sugar alcohols, and amino acids. They are found in many types of plants and are frequently utilized by insects as stimulants for feeding (32 -33). The presence of a sugar-sensitive cell in every species tested (5,12, 19, 34) demonstrated that Lepidoptera respond strongly to sucrose as a phagostimulant (35). Flower nectar provides a high-carbohydrate food source for the majority of adult Lepidoptera, and it is required for the female to breed (36-37).

Feeding and food perception are integrated into the proboscis extension response, which occurs when their antennae touch a sugar solution (38). The behavior of the proboscis extension reflex (PER) in herbivorous insects can be used to track their responses to feeding signals. When phagostimulants

were applied to the antennae of noctuid moths, they released PER, whereas deterrents reduced it (37,39). Sucrose-elicited PER has been described and implicated in associative learning in a variety of restrained insects, including moths (39-41), butterflies (42), and bees (43,44). To comprehend an insect's feeding and oviposition behavior, first attempt to understand the chemosensory code that underpins these behaviors (45). There is currently little known about how the GSNs on the antennae of the female *S. littoralis* perceive oviposition deterrents found in the conspecific larval frass or oviposition stimulants in the surface phytometabolites of their preferred hosts' green leaves. Moths therefore depend on their gustatory sense to judge the quality of their food and distinguish between what is toxic and what is edible (46,48).

Though Lepidoptera is a large order of insects, little is known about antennal taste recognition in this group of insects. The presence of antennal gustatory sensilla is reported in a limited number of Lepidopteran species (i.e. 49-50). Therefore, it is still unclear whether the function of the antennal gustatory sensilla is common in Lepidoptera. The objectives of this study were to examine the response profiles of the antennal sensilla identified in two separate sites at the same flagellomere of the antenna of the female *S. littoralis* to diverse stimuli. The current study also focused on compounds assumed to be important in the selection of the plant hosts for feeding or oviposition, which will provide insights into the mechanisms and functions of GSNs to optimize current attractants or deterrents. Understanding insect taste may help develop new or more effective pest control methods as well as our understanding of how insects view their surroundings (51).

Methods and materials

Insects

Females *S. littoralis* utilized in tests were grown in a laboratory. The first set of larvae was grown on fresh cotton leaves, *Gossypium barbadense* Mill (Malvales: Malvaceae), for frass collection. The second set of larvae was fed on an artificial diet and reared for electrophysiological testing. All moth developmental phases were preserved at 25°C, 70% R.H, and LD 16:8 h. Pupae were collected, sexed, and kept apart until they emerged to be used in experiments.

Scanning electron microscopy

Antennae were cut off with micro scissors and left in 70% ethanol overnight at 4 C in preparation for scanning electron microscopy (SEM). Afterward, the specimens were dehydrated in ethanol at 80%, 90%, and 100% concentrations. They were then placed on SEM stubs and coated with gold-palladium (3:2) using a JEOL ion sputter JFC-1100. Using a scanning electron microscope (LEO 435 VP, UK), the specimens were made visible. From SEM micrographs of a distal segment of the antenna, each morphological sensillum type of the gustatory sensilla chaetica was investigated.

Electrophysiology

Preparation of chemical stimuli

Frass was collected from fourth and fifth late-instar larvae feeding on cotton plant leaves, *G. barbadense*. After collection, the frass was stored in dark, airtight conditions at -20°C.

According to preliminary studies (52), it was found that the extract of 15 mg frass/ml ethanol significantly deterred the oviposition of the female *S. littoralis* in a behavioral assay. Therefore, frass extracts were prepared as follows: 2.25 g frass was added to 250 ml absolute ethanol, stirred for 24 h at room temperature, and then filtered with a filter paper (Whatman No. 1) to obtain an ethanol extract. The residue was vacuum-dried to finally obtain a stock of 150 ml extract at the concentration of 15 mg frass/ml ethanol.

The surface phytochemicals of green cotton leaf extract were obtained by immersing each complete green leaf in the solvent for 2 seconds after washing 100 g of fresh green cotton leaves (8 weeks old) in 150 ml absolute ethanol. The extract was filtered

using Whatman No. 1 filter paper and vacuum-dried to yield a stock of 100 ml ethanol extract with a concentration of 1.0 g cotton leaves extract/ml ethanol.

Stimuli

To functionally characterize the differential responses of the gustatory sensilla Vch and Lch present on each antennal segment of the distal third of the antennal flagellum of the female *S. littoralis* with host and non-host chemical stimuli, 10 mM and 100 mM sucrose; 10 mM NaCl; 1% and 10% ethanol prepared in dilutions of the electrolyte (10 mM NaCl), 1% and 10% crude frass extract (15 mg/ml); and 10% crude extract of green cotton leaves (1 g/ml). All stimuli were dissolved in a double-distilled water solution of 10 mM NaCl. An electrolyte solution of 10 mM NaCl was utilized.

Electrophysiological recordings

The moths were held in a 1 ml plastic, disposable pipette tip holder, with one of their antennae extending from the pipette tip. The exposed head was immobilized with dental wax to hinder the moth from moving. The mounted insect was then placed on a microscope slide, and the antenna was attached to an elevated cover slip with double-sided sticky tape. A tungsten wire (diameter 0.12 mm, Harvard Apparatus Ltd, Edenbridge, United Kingdom) was inserted into the insect's abdomen as a reference electrode. Sensilla could be seen at high magnification (750x). The tip recording technique was used for electrophysiological recordings (53). Borosilicate glass capillaries (1 mm outer diameter x 0.75 mm inner diameter) were used to make electrodes with a tip diameter of 20 µm. Just before the recording began, the recording electrode was filled with the test or control stimuli. The electrode was subsequently attached to a taste probe (Syntech, Kirchzarten, Germany), which allowed for reliable AC recordings from the GSNs contained in each sensilla. The taste probe was coupled to an amplifier (Syntech Taste Probe DT-02) with automatic offset adjustment (54). Electrical impulses were amplified and filtered (bass band filter: 100-1000Hz) using an analogue to the digital signal converter (IDAC, Syntech), which was connected to a PC computer for signal recording and visualization. The recording electrode was placed over an individual sensillum using a micromanipulator. To avoid adaptation, stimulation was given for 2 to 3 seconds with a 5-minute interval between stimuli. To analyze responses to varied stimuli, the total number of spikes recorded within the first second of a recording was employed. Data were acquired from the Vch and Lch sensilla chaetica of 22 distal antennal segments of ten females (Figure 1). Because of the small number of insects studied, the results should be regarded as qualitative, as a comprehensive physiological characterization of a diverse array of antennal sensilla was not possible. Understanding the molecular basis of polyphagy may provide opportunities for the development of new environmentally friendly pest control strategies, like push and pull, and develop Integrated Pest Management programs.

The behavior of proboscis extension reflex

Proboscis extension reflex, PER, was assessed to varying concentrations of sucrose touching the antennae of the female *S. littoralis* to test if the antennal gustatory sensilla is responsive to sucrose (n=45). Gustatory response data were binomial, meaning that the moth either responded with PER or did not (1 or 0). Phagostimulant sucrose was applied to the tip of the antennae for these tests. One-day-old moths were starved for two days before being held in Plexiglas tubes and assessed. When the insect expanded its proboscis 1-5 seconds after the stimulant contacted the antennae, this was considered positive PER behavior. When the insect did not expand its proboscis following stimulation and responded with antennae withdrawal, PER was extinguished. All experiments were replicated three times with 15 females tested in each experiment. Water was used as a control, followed by 1 mM, 10 mM, 100 mM, and 1000 mM sucrose. To avoid adaptation, a 7-minute intertrial interval between

stimulation was conducted. The PER with all tested solutions was scored and compared.

Statistical Analysis

Action potentials (spikes) of three GSNs could be distinguished in each sensillum and were manually classified as 'N1', 'N2', and 'N3' neurons based on differences in spike amplitudes and waveforms, with 'N1' having the largest spike amplitude and 'N3' having the smallest.

A one-way ANOVA followed by a Tukey-Kramer Multiple Comparisons Test was used to assess the responses of the responsive GSNs associated with two separate sensilla types: Vch and Lch, to different tested stimuli (GraphPad InStat, Inc., California, USA).

Results

Electrophysiological recordings were made from two different types of antennal gustatory sensilla chaetica according to their location on each antennal segment. The first type of sensilla was the ventral one (Vch) located on the ventral side of the antennal segment and the second type was the lateral sensillum (Lch) located on the ventrolateral side (Figure 1). Electrophysiological recordings were made from ~100 sensilla of each type (Vch or Lch) found on ten consequent antennal segments of the distal third of the antenna of 10 virgin females S. *littoralis* (Figure 1 B and D). The GSNs exhibited excitatory phasic-tonic multicellular neuronal responses of variable magnitude in response to the different tested stimuli (Figures 2-3 and 5-6). While in some cases, inhibitory responses were also recorded.

Stimulation of Vch and Lch sensilla with 10 mM NaCl showed similar characteristic firing patterns in N1 and N2 neurons (Figures 2-6). the higher concentration of sucrose, in general, evoked, unlike responses in N1 and N2 neurons of both types of sensilla (Table. 1). However, the N3 neuron was notably stimulated only with a higher concentration of sucrose, ethanol extracts of green cotton leaves and conspecific larval frass extract (Figures 2-3).

Responses of Vch sensilla

In Vch sensilla, stimulation of them with 10 mM NaCl elicited responses in neurons N1 (27.4 ± 6.7 imp/s) and N2 (2.4 ± 1.4 imp/s) (Figure 3). However, stimulation with 10 mM or 100 mM sucrose evoked a phasic-tonic significant inhibition in the activities of N1 neuron (7.9 ± 3.2 imp/s and 3±0.8 imp/s, respectively) compared with its activity with 10 mM NaCl (one way ANOVA, $q=4.33$, $P<0.01$ and $q=9.38$, $P<0.001$, respectively). Moreover, the N2 neuron was activated with 10 mM and 100 mM sucrose (Table. 1). Activation of the N2 neuron (34 ± 5.7 imp/s) with the higher concentration of sucrose (100 mM) was not significantly different than those recorded with the lower concentration of sucrose (10 mM) (one way ANOVA, $P>0.05$). While the N3 neuron was observed to be only activated with the higher concentration of sucrose (5.3 ± 1.8 imp/s) (Figure 4).

Stimulation of Vch sensilla with 1% ethanol evoked inhibition of N1 neuron (12.25 ± 3.03 imp/s) and activation of N2 neuron (30.9 ± 7.07 imp/s) (Figure 2). In contrast, higher concentrations of ethanol (10%) activated N2 neuron with burst firing responses (53.7 ± 8.3 imp/s). the response of N2 neuron recorded with 10% ethanol was significantly different than this with 1% ethanol (one-way ANOVA, $q=4.38$, $P<0.05$).

Noticeably, stimulation of Vch sensilla with 10% ethanol extract of green cotton leaves highly activated N3-neuron (30 ± 3.88 imp/s) (Figure 5). In addition, a significant deactivation of the N1 neuron was recorded (4.33 ± 2.53 imp/s), which was significantly different than its activation with 10 mM NaCl (one-way ANOVA, $q=6.24$, $P<0.001$). On the other hand, compared to their responses with the electrolyte, the activity of N2 neuron with 10% green cotton leaf extract was non-significantly different (3.3 ± 0.79 imp/s) (Figure 5-6). Stimulation of Vch sensilla with ethanol extracts of the conspecific larval frass (1% or 10%) totally inhibited action potentials of the N2 neuron and activated N3 neuron (Figure 5). However, the higher concentration of the frass extract (10%) significantly

suppressed the activation of N3 neuron. Furthermore, complete inhibition of all responsive GSNs associated with Vch sensilla was observed in ~40% of the tested sensilla when stimulated with 1% or 10 % frass extracts.

Responses of Lch sensilla

Stimulation of Lch sensilla with 10 mM NaCl elicited responses in N1 (20 ± 3.32 imp/s) and N2 (1.2 ± 0.2 imp/s) neurons (Figure 3). In the case of Lch sensilla, a different pattern of responses than those recorded with Vch sensilla was observed. Stimulation of Lch sensilla with 10 mM sucrose elicited a similar characteristic firing pattern of N1 and N2 neurons (N1: 12.7 ± 3.8 imp/s and N2: 30.4 ± 5.8, respectively) as those associated with Vch sensilla (Figure 4). However, a different pattern of response has been recorded with a higher concentration of sucrose (100 mM) (Table 1). The N1 neuron was observed to be highly activated with 100 mM sucrose (29.9 ± 3.16 imp/s) than with 10 mM sucrose (one-way ANOVA, $q=5.53$, $P<0.01$). But a significant deactivation of the N2 neuron with the higher concentration (100 mM) of sucrose was observed (10.7 ± 1.09 imp/s) compared with its activation with the lower concentration of sucrose (10 mM) (one way ANOVA, $q= 4.8$, $P<0.05$).

Compared to 10 mM NaCl, 1% ethanol did not significantly activate the N1 neuron (17.3 ± 3.54 imp/s) (one-way ANOVA, $q=0.815$, $P>0.05$), but it was significantly deactivated with the higher concentration of ethanol (10%) (one way ANOVA, $q=4.97$, $P<0.05$) (Figure 3). The lower concentration of ethanol (1%) elicited a moderate activation of N2 neuron (10.4 ± 0.96 imp/s) (Table. 1). A different pattern of response has been recorded with the higher concentration of ethanol (10%), i.e., a significant higher firing frequency of N2 neuron (33.3 ± 5.67 imp/s) with a burst firing have been recorded compared to its activity with the electrolyte (one way ANOVA, $q=5.48$, $P<0.01$) and a higher deactivation of N1 neuron was observed (4.7± 2.11 imp/s) (Figures 3-4).

Typically, stimulation of Lch sensilla with ethanol extracts of the green cotton leaves or conspecific larval frass elicited a deactivation of N1 neuron (Figure 6). However, a higher deactivation of N2 neuron (3.3 ± 0.79 imp/ s) with 10% extract of green cotton leaves than this with 10% ethanol alone was also recorded (one-way ANOVA, $q=4.99$, $P<0.05$). However, a complete inhibition of N2 neuron with 1% and 10% frass extracts was observed (Figure 6).

The N3 neuron was notably activated with 10% green cotton leaves extract (35.4 ± 7.02 imp/s) and 1% frass extract (17.4 ± 2.3 imp/s) (Figures 5-6). Whereas the firing frequencies of N3 neuron with 10% green cotton leaves extract or 1% frass extract were significantly higher than those with 10% frass extract (2.33 ± 0.87 imp/s) (one way ANOVA, $q=4.83$, $P<0.05$; and $q=5.37$, $P<0.01$, respectively). In ~50% of traces complete inhibition of activities of all neurons associated with Lch sensilla with frass extracts was observed.

Typically, stimulation of Vch or Lch sensilla with ethanol alone evoked distinct responses other than those with the same concentration of extracts of green cotton leaves or conspecific larval frass. Whereas peripheral interaction between ethanol and crud extracts has been observed (Table 1).

Behavioral PER responses after stimulation of antennal GSNs

The hypothesis that antennal gustatory sensilla have receptor cells responding to water and sugar and differ in their responses to different concentrations of sugar has been tested (Figures 7-8). It has been discovered that the female *S. littoralis's* gustatory responsiveness, as indicated by the robust extension of her proboscis (PER), was dose-dependent at all tested concentrations (Figure 8). The first trial of stimulation of antennae with distilled water elicited a positive PER in 25.3% of the female moths. When appetitive sucrose was then applied, the PER proportions were increased with increasing the concentrations (1 mM, 10 mM, 100 mM, and 1000 mM) scored (35.2%, 39.2%, 58.6%, and 84.4%, respectively) (Figure 8).

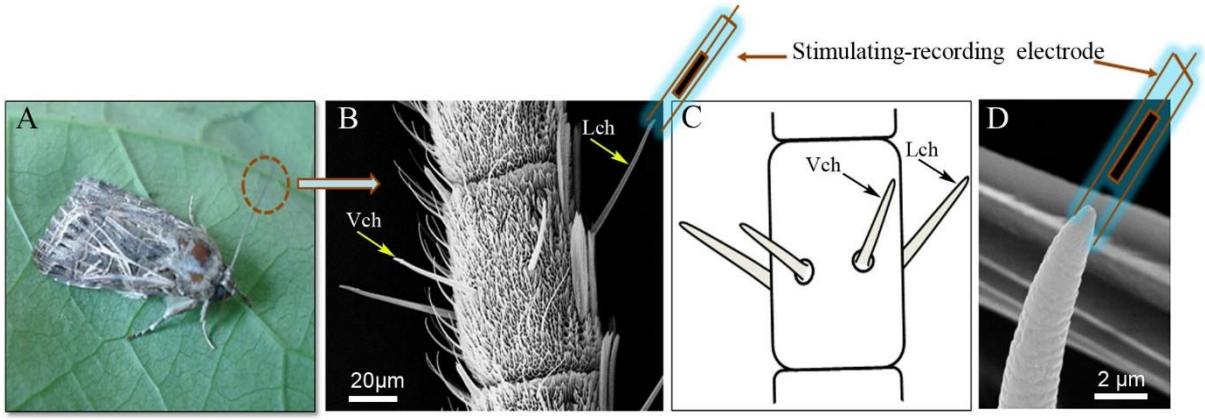

Figure 1.
Recording technique and morphology of the antennal gustatory sensilla chaetica of the female *Spodoptera littoralis*. (A) An adult female, with the recording area denoted by a dotted circle, encircling the distal third of the antenna. (B) Scanning electron micrographs (SEM) of one distal segment of the antenna of the female with two types of gustatory sensilla chaetica (Vch and Lch); scale bar, 20 μm. (C) Schematic diagram of the ventral view of one antennal segment representing the position of Vch and Lch sensilla chaetica. (D) Higher magnification of Vch gustatory sensillum the apical pore of sensilla was at the tip of the sensillum; scale bar, 2 μm. (Note: During recording, the sensillum's tip was capped by the stimulating-recording electrode as shown in B and D).

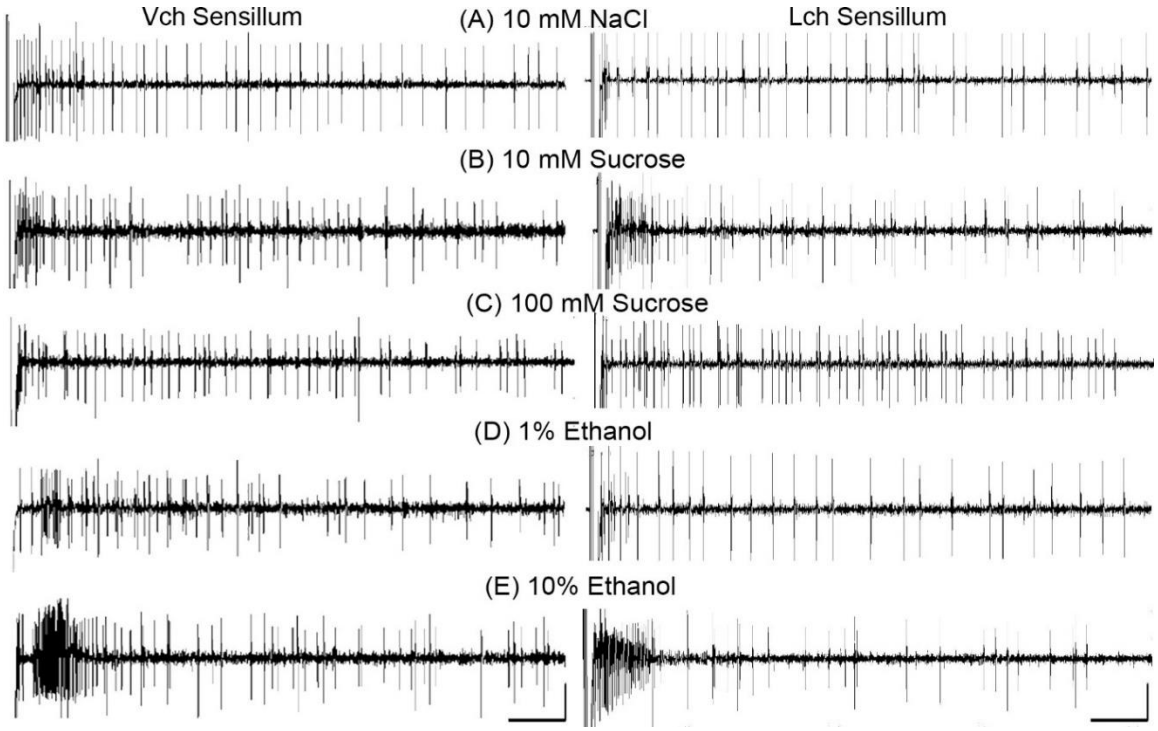

Figure 2.
Example recordings from the Vch and Lch gustatory sensilla chaetica on the distal third of the antenna of the female *Spodoptera littoralis* to (A) 10 mM NaCl (control), (B) 10 mM sucrose, (C) 100 mM sucrose, (D) 1% ethanol and (E) 10% ethanol; time panel in all traces = 2 s. Note the patterns of responses of Vch sensilla were the activation of Neuron 1 (N 1) with NaCl and higher deactivation of it with 100 mM sucrose; activation of Neuron 2 (N 2) with sucrose, and higher activation of it with 1% and 10% ethanol, and weak activation of Neuron3 (N 3) with electrolyte and 100 mM sucrose. In Lch sensilla, the patterns of responses were the activation of Neuron 1 (N1) with NaCl and higher activation of it only with 100 mM sucrose; activation of Neuron2 (N2) with 10 mM sucrose, and higher activation of it with 10% ethanol, and weak activation of Neuron3 (N 3) with electrolyte (vertical bar, 1mV; horizontal bar, 100ms).

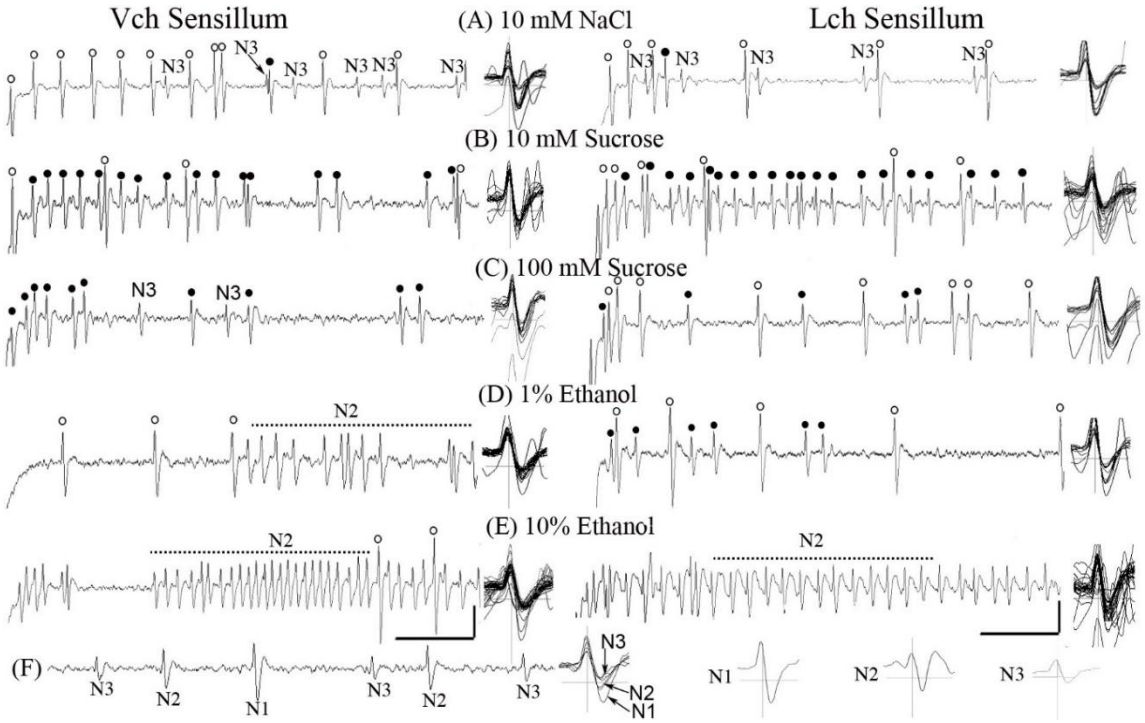

Figure 3.

Multi-unit action potential activity recorded from two types of sensilla chaetica (Vch and Lch) on the distal third of the antenna of female *Spodoptera littoralis*, using the extracellular tip-recording technique (right) and wave forms of the responding neurons in 200ms (left to each trace). (A) Response to 10 mM NaCl (control). (B) Response to 10 mM sucrose, Neuron 2 being the dominant active neuron. (C) Responses to 100 mM sucrose, presenting a favorable example, in which activity of three neurons (Neurons 1, 2 or 3) can be distinguished in Vch sensilla, Neuron2 being the most active in Vch sensilla but N1and N2 neurons of Lch sensilla were activated. (D) Responses to 1% alcohol, in which activity of two neurons is observed, Neurons 1 and 2. (E) Responses to 10% alcohol, in which activity of N2 neuron is observed with burst firing (dotted upper line) in both types of sensilla, Vch and Lch, and inhibition of N1 Neuron. (F) The activity of the gustatory sensory neurons (GSNs) housed in the antenna s. chaetica reveals differences in spike amplitude between Neurons 1–3 (the spike shapes and amplitudes of the three neurons are shown). Each action potential is labeled with either 1, 2, or 3. Upper traces in (A)–(E) represent 0–200 ms, opened circles represent N1 Neurons, and Blocked circles represent N2 Neurons (vertical bar, 1mV; horizontal bar, 50ms). All responses from (A)–(E) are of the same antenna preparation.

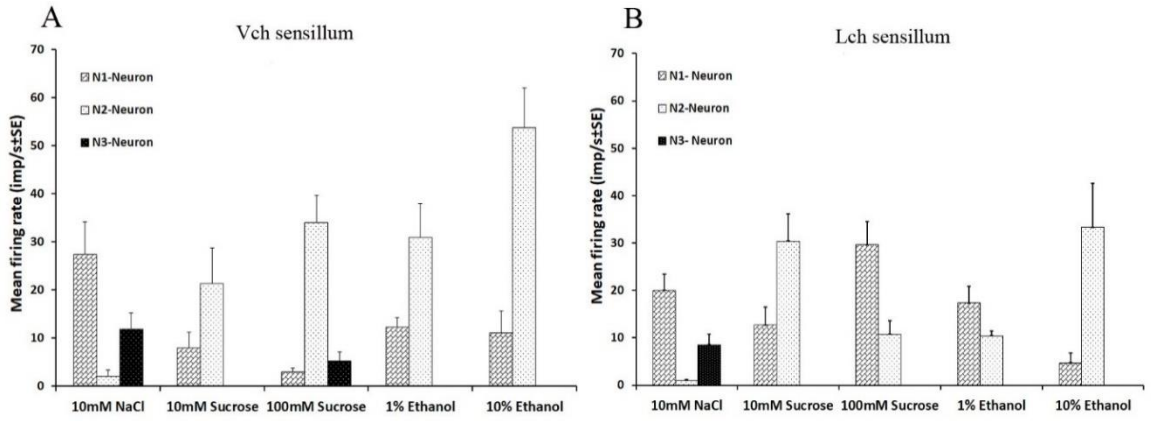

Figure 4.

Electrophysiological responses (mean ± SE imp/s) of the responding gustatory sensory neurons (GSNs) of Vch and Lch sensilla chaetica of the distal third of the antenna of the female *Spodoptera littoralis* to 10 mM NaCl, 10 Mm and 100 mM sucrose, 1% and 10% ethanol. Action potentials were counted for 1 s after the onset of the stimulus (stimulus onset artifact lasting about 10–15 ms). Vertical bars show the SE of the means ($20 \leq n \leq 40$).

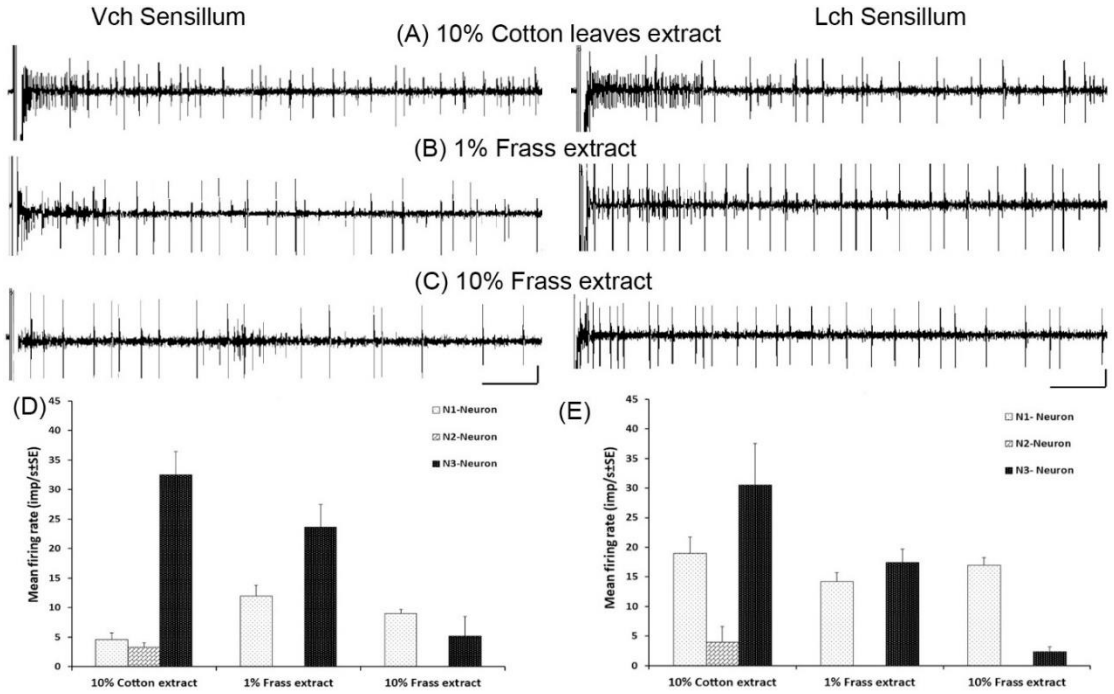

Figure 5.

(A-C) Sample recordings from Vch and Lch sensilla chaetica stimulated with 10% ethanol extract of green leaves of cotton, 1% and 10% ethanol extract of the conspecific larval frass, showing that N3 neuron was mainly activated with 10 % ethanol extract of green cotton leaves and 1% ethanol extract of the conspecific larval frass; and deactivation of it with the higher concentration of frass extract (10%). Note the deactivation of N1 neuron with 10% extracts of green cotton leaves and conspecific larval frass. Time panel in all traces = 2 s. (D-E) Electrophysiological responses (mean ± SE imp/s) of the responding gustatory sensory neurons (GSNs) within Vch and Lch sensilla chaetica of the distal third of antennae of the female *Spodoptera littoralis* to 10% ethanol extract of green cotton leaves, 1% and 10% ethanol extract of conspecific larval frass. Action potentials were counted for 1 s after the onset of the stimulus. Vertical bars show the SE of the means ($20 \leq n \leq 40$).

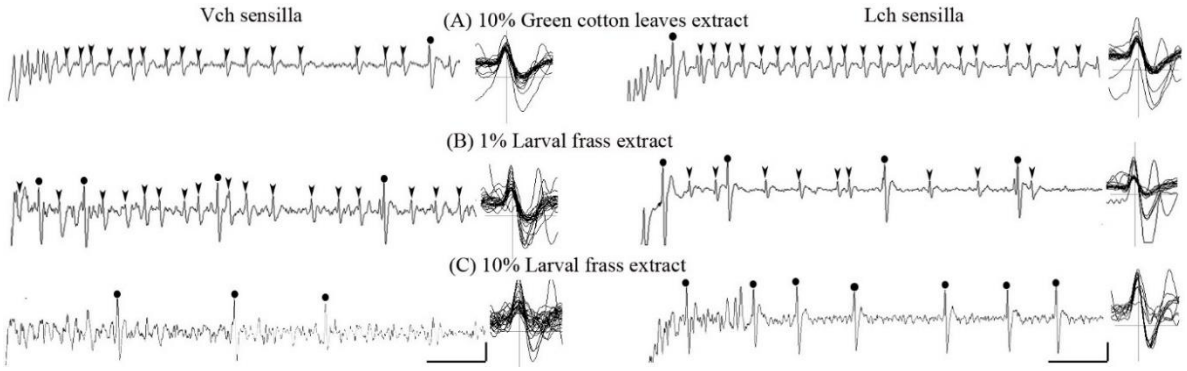

Figure 6.

Multi-unit action potential activity was recorded from two types of sensilla chaetica (Vch and Lch) on the distal third of the antenna of female *Spodoptera littoralis*, using the extracellular tip-recording technique (right) and waveforms of the responding neurons in 200ms (left to each trace). (A) 10% ethanol extract of green leaves of cotton, Neuron 3 being the dominant active neuron with burst firing activity (black arrowheads). (B) Response to 1% ethanol extract of the conspecific larval frass, Neuron 3 being the dominant active neuron (black arrowheads). (C) Response to 10% ethanol extract of conspecific larval frass and inhibition of N2 and N3 Neurons were observed. Each action potential is labeled with either 1, 2, or 3. All traces in (A)–(C) represent 0–200 ms (vertical bar, 1mV; horizontal bar, 50ms). All responses from (A)–(C) are of the same antenna preparation.

Table 1. Electrical identification of three gustatory sensory neurons GSNs (N1-N3) of the ventral (Vch) and lateral (Lch) sensilla cheatica associated with the distal third of antenna of the female *Spodoptera littoralis* according to spike amplitudes in response to 10mM NaCl, 10mM sucrose, 100mM sucrose, 1% ethanol, 10% ethanol, 10% cotton extract, 1% and 10% conspecific larval frass extracts, respectively.

Tested compound	**Responsive Neurons**	
	Vch sensillum	Lch sensillum
10 mM NaCl	$N1^{++}$ $N3^{+}$	$N1^{+}$ $N3^{+}$
10mM Sucrose	$N1^{-}$ $N2^{++}$	$N1^{\pm}$ $N2^{++}$
100mM Sucrose	$N1^{-}$ $N2^{+}$	$N1^{++}$ $N2^{+}$
1% Ethanol	$N1^{-}$ $N2^{++}$	$N1^{\pm}$ $N2^{+}$
10% Ethanol	$N1^{-}$ $N2^{+++}$	$N1^{-}$ $N2^{+++}$
10% Cotton leaves extract	$N1^{--}$ $N3^{+++}$	$N1^{--}$ $N3^{+++}$
1% Frass extract	$N1^{-}$ $N3^{++}$	$N1^{\pm}$ $N3^{+}$
10% Frass extract	$N1^{--}$ $N3^{-}$	$N1^{\pm}$ $N3^{-}$

Different signs precede each distinct neuron referring to activation of this neuron ($^{+}$: 5-10 spikes, $^{++}$: 11-20 spikes, $^{+++}$: >20 spikes), deactivation of the neuron ($^{-}$:5-10 spikes, $^{--}$: 11-20 spikes), and the same response of the neuron ($^{\pm}$: < 5 spikes of activation or deactivation) with tested stimuli compared with their responses with 10 mM NaCl.

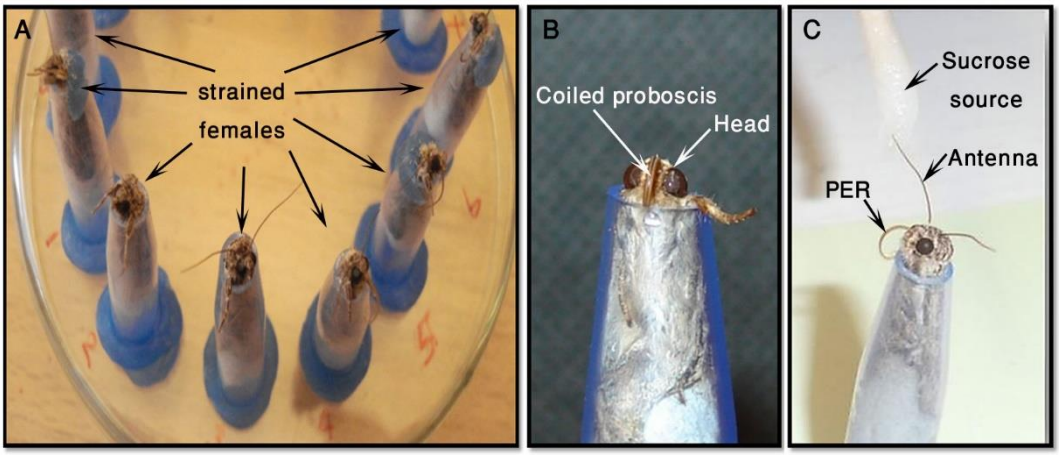

Figure 7.

(A) Females *Spodoptera littoralis* restrained in Plexiglas tubes before being tested. (B) Restrained female with coiled proboscis at rest before stimulation. (C) Positive PER behavior by extending the proboscis 1-5 s after the stimulant (sucrose) contact with the antennae.

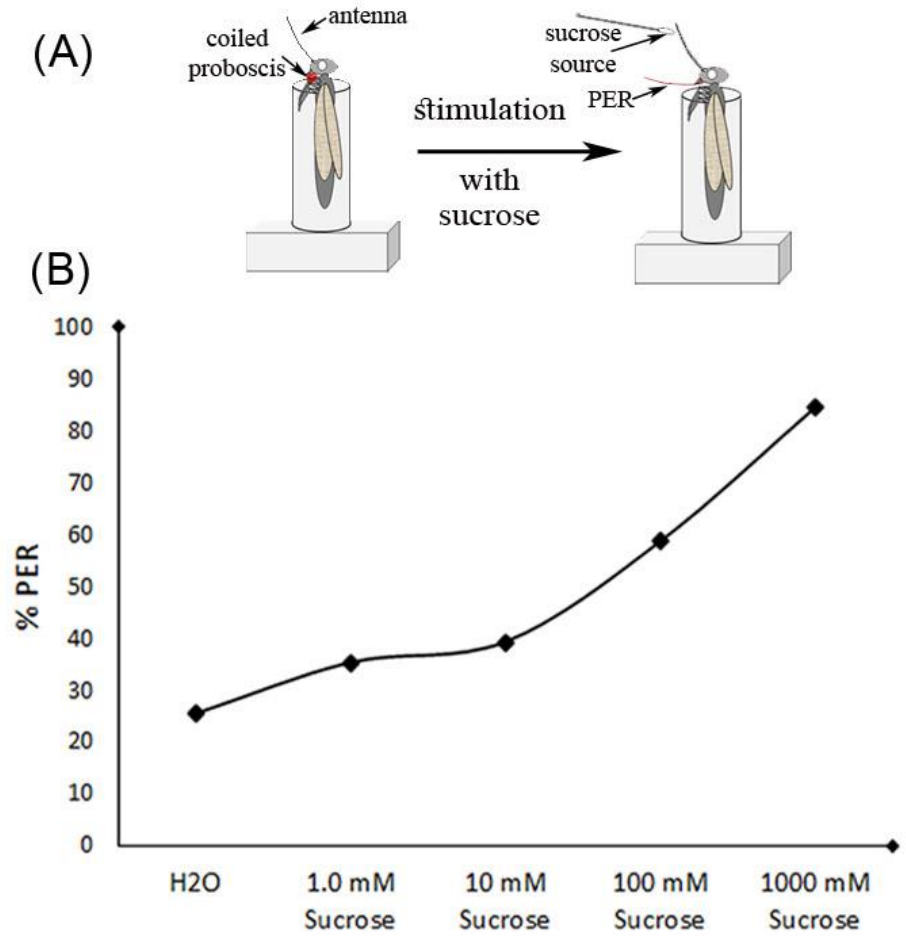

Figure 8.

Sucrose responsiveness by the female antennae of *Spodoptera littoralis*. (A) Diagrammatic design of the experiment before and after delivering the stimulant (sucrose). (B) The dose-response curve for various sucrose concentrations (x-axis); the y-axis reports the PER rate, i.e., the proportion of insects extending their proboscis when their antennae were briefly touched with a drop of sucrose solution. Each sucrose solution presentation was separated by 10 min.

Discussion

Electrophysiology

Understanding how animals utilize their surroundings to control their behavior is a key task in neuroscience. This study provides evidence for the gustatory function of moth's antennae. Additionally, notable distinctions between the locations of sensilla and stimuli have been discovered. According to Mitchell et al. (25) and Hallberg (24), the flexible socket at the base of these sensilla chaetica suggests a mechanosensory function. Usually, observations supported this function (data not shown). Due to a lack of reports of uniporous sensilla chaetica on the antennal flagellum of *S. littoralis*, it was unsure of the extent of gustation in the antennae of Lepidoptera with putative host gustatory cues. Sensilla recordings were unvague and clear with no difficulties when using a well-mounted antenna. On the other hand, inconsistent contacts made it difficult for Amat et al. (6) to obtain electrophysiological recordings from sensilla chaetica of the labial palps and antenna of adult moths of *Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis and

Shiefermüller). To date, *Drosophila* has gotten far more attention in insect taste investigations than Lepidoptera species, which contain many dangerous agricultural crop pests (55).

In the present study, it has been confirmed that different tastants like sugar, salt, alcohol, and leaf surface phytochemicals, deterrent compounds like conspecific larval frass extract evoked different electrophysiological responses in the GSNs of the antennal sensilla chaetica of the female *S. littoralis*. The perception of biologically active ligands of either insect or plant origin by antennal gustatory sensilla of the female moth *S. littoralis* has remained until now mostly unexplored, despite its importance in the final steps of the selection of the host plant for feeding or oviposition.

In the present study, the majority of the gustatory sensilla of the antennae of the female *S. littoralis* displayed multineuronal responses, indicating that gustatory coding on the antenna of the female *S. littoralis* comprises multiple channels. Female moths use their antennae for olfaction as well as tasting the host plant surface before egg deposition by drumming their antennae with the substrate. This drumming motion most likely causes phytochemicals in the leaf wax layer that signal plant quality to come into direct contact with gustatory sensilla, which are particularly plentiful near the antennal tip (15.18.56).

The current data showed that the GSNs of Vch and Lch gustatory sensilla of the female *S. littoralis* antennae have distinct response profiles to single compounds, such as NaCl, sucrose, and ethanol, as well as mixtures of compounds, such as crud extracts of green cotton leaves and conspecific larval frass. Furthermore, their differential responses due to their diverse placement at the same antennal segment were obtained. The temporal responses of the two major responsive neurons in both types of sensilla, N1 and N2, were similar with the lower dose of sucrose (10 mM). The increased concentration of sucrose (100 mM) induced unique responses in two different neurons of both types of sensilla, activation of N2 neuron and inhibition of N1 neuron of Vch sensilla. In contrast, inhibition of the N2 neuron of Lch sensilla and activation of the N1 neuron have been recorded. Furthermore, activation of the N3 neuron in the Vch sensilla was only observed with the higher concentration (100 mM) of sucrose. These findings suggested that Lch sensilla had two unique receptor cells that respond to either low or high sucrose concentrations, which could result in a varied behavioral output.

In contrary to what has been observed in this study, Amat et al., (6) found that the activity of the antennal sensilla of three tortricid moths (Lepidoptera: Tortricidae) decreased when sugar concentration increased. Many noctuids are flower visitors, so different sugar requirements between members of different lepidopteran families may explain differences in sugar sensitivity. Similar observations to this study were recorded in *Heliothis virescens* (Fabricus) and *Spodoptera littoralis* (Boisduval) (31.39).

Sugars and salts have an impact on the moths' behavior, longevity, and level of fitness (6). Sugars encourage oviposition in *C. pomonella* and *L. botrana* (57.58). Gustatory sensilla in the ovipositor and tarsi of *S. littoralis* and ovipositor of *L. botrana* identify fructose and sucrose (5.19.58). Additionally, the fitness of larvae may be impacted by salts and sugars (59. 60. 61). Additionally, the antennal GRNs of adult Lepidoptera respond to water, salts, sugars, amino acids, and bitter substances (6.31.39.62). Furthermore, Mu et al., (63) could identify sensory neurons responsive to sucrose, glucose, nicotine, and tannic acid in the medial and lateral sensilla styloconica of the larvae of the potato tuber moth, *Phthorimaea operculella*. However, earlier studies on the styloconica gustatory sensilla on the maxillae and proboscis of adults and larvae of *S. littoralis*, *H. virescens*, and *Helicoverpa armigera* revealed differential responses to different types of sugar rather than to one type of sugar (29).

The current findings are consistent with an earlier study that has investigated variances in the responsiveness of GSNs based on their spatial position. Jörgensen et al. (39) discovered changes in the response of *H. virescens* antennal gustatory sensilla to sucrose based on their location on the tip or at the base of the flagellum, but not in the same flagellomere. Similarly, Liscia et al. (64) discovered that the sensitivity of *Protophormia terraenovae* labellum sensilla to sugars differed depending on sensillum type and location. Popescu et al. (31) also found that the responsiveness of the female *S. littoralis* antennal gustatory sensilla to three sugars and two inorganic salts did not vary significantly along the antenna. Calas et al. (17) explored the association between insect tarsal gustatory sensilla sensitivity and location. Similarly, Seada et al. (19) identified three distinct functional groups of the female *S. littoralis* tarsal gustatory sensilla based on their spatial placement.

Surprisingly, the lower concentration of ethanol activated the same neurons (N1 and N2) that responded to the lower concentration of sucrose in both types of sensilla (Vch and Lch). Higher concentration of ethanol elicited greater activation of N2 neurons with a burst firing pattern in both types of sensilla, Vch and Lch. The current findings are somewhat comparable with the findings of Jörgensen et al. (39), who claimed that ethanol is fat soluble and may act directly on the GSN membranes, inducing significant amplitude spikes of antennal gustatory sensilla in the female moth *H. virescens*. Furthermore, in the present study, both lower and higher concentrations of ethanol activated the sugar neuron (N2) of Vch and Lch sensilla. Similarly, recordings from monkey chorda tympani nerves showed that ethanol stimulates sweet-best fibers and at high concentrations some salt-best fibers too (65). In contrast, ethanol did not elicit responses in the sucrose-sensitive neurons of the antennal gustatory sensilla of the female moth *H. virescens* (39). One of the most prevalent major plant metabolites is ethanol (66). As a result, female moths may contain ethanol-responsive neurons that allow them to accept or reject suitable host plants for feeding or oviposition. Thibodeau and Pickering (66) suggested that ethanol is often unpleasant since it causes bitterness and discomfort when consumed. Jörgensen et al. (39), on the other hand, discovered that ethanol was attractive to *H. virescens* larvae.

The current findings showed that a crude extract of green cotton leaves inhibited N2 neuron activity in both types of sensilla (Vch and Lch). However, this neuron was activated more when the solvent (10 % ethanol) was used alone. Thus, a mutual inhibition of ethanol responsive neuron (N2) could be owing to the inhibitory impact of the secondary metabolites detected in cotton leaf extract as a defensive mechanism of the cotton plant against herbivores. Similarly, Xu et al. (67) discovered that a crude extract of cotton leaves might activate three types of deterrent gustatory receptors in the polyphagous moth *Helicoverpa armigera*. Interestingly, in the present study, the crude extract of green cotton leaves significantly stimulated the N3 neuron. The presence of a receptor cell (N3) in the antenna of the female *S. littoralis* responding to token stimuli found in the crude extract of green cotton leaves in both Vch and Lch sensilla, which is responsible for the identification of their host-related compounds, is possibly the most intriguing speculation that emerged from this result. The current data also demonstrated that the crude extract of the conspecific larval frass exhibited a moderate inhibitory response of the N1 neuron, complete inhibition of the N2 neuron, and burst firing of the N3 neuron. A higher dose of crude extract of conspecific larval frass inhibited both N1 and N3 neurons. In contrast, the N1 neuron of Lch sensilla responded to lower concentrations of the frass extract in the same way that it did to 10 mM NaCl, whereas increasing the concentration of the crude frass extract did not elicit any changes in the action potentials of N1 neuron, but deactivation of N3 neuron was observed. These findings could imply that the antennal sensilla of female *S. littoralis*

includes a host plant recognition receptor cell (N3), both tested doses of the frass extract inhibited all responding neurons in 45-50 % of the tested sensilla. The activation of N3 neuron with crude cotton extract and only at lower concentrations of the frass extract could be explained by this neuron's specificity as a host-specific receptor neuron. Because the frass was collected from larvae fed on green cotton leaves, crude extracts of green cotton leaves and larval frass share certain host plant components. Whereas activating it may cause female moths to recognize the host plant as a token stimulus, the deactivation of it in the presence of a larger quantity of larval frass extract may prevent them from ovipositing. Similarly, Yang et al., (68) discovered that a deterrent sinigrin was a potent feeding stimulant, eliciting activity in both larval maxillary sensilla and adult medial tarsal sensilla of the cabbage butterfly *Pieris rapae*. It is, however, a secondary plant metabolite that is used as a cue in the host selection of several crucifer specialist insects.

The bulk of secondary plant metabolites act as protective compounds against herbivorous insect attacks by inhibiting feeding and oviposition. Some monophagous and oligophagous insects have an intriguing adaptation in which they use these compounds as token stimuli to recognize host plants for feeding or oviposition (32.68-69). It has been demonstrated that chemical messengers of either insect or plant origin alert females about the earlier infestation of host plants with their conspecific species in a wide range of phytophagous insects (22). Feeding larvae and expelled larval frass in Lepidoptera indicate earlier occupancy of the host plant and discourage egg laying (70-71). Early research discovered that crude extracts of conspecific larval frass of *S. littoralis* inhibit oviposition in gravid moths (23.72). Furthermore, Hilker and Klein (23) discovered that oviposition-deterring chemicals present in *S. littoralis* larval frass were behaviorally recognized by the female's antennae. Despite the limitations of the crude extracts employed to test the electrical responses of *S. littoralis* antennal gustatory neurons to host and non-host gustatory stimuli. Surprisingly, response patterns revealed that antennal gustatory sensilla host gustatory neurons, are potential receptors for host-plant recognition. Furthermore, because the crude extracts of green cotton leaves and conspecific larval frass contain unknown ingredients, it is impossible to make inferences on the precise tuning specificity of the GSNs in response to tastants derived from various basic taste categories as crude extracts. To explain the events that precede host selection by the female moth, it would be extremely challenging to determine the perception of mixtures of putative physiologically relevant ligands of plant or insect origin using behavioral, analytical, and electrophysiological studies.

Behavioral PER responses

Behavior is the outward manifestation of neuronal processes that are impacted by both physiological and environmental factors. Understanding the nervous system's limitations and functioning is critical for understanding herbivorous insect feeding behavior and establishing efficient pest management measures for *S. littoralis* (73). Adult moth feeding behaviors and gustatory perception are critical for reproduction but are underutilized in pest management (74-79).

The sugar response assay is straightforward, but it provides a wealth of experimental possibilities. To detect sugars and manage these diverse behaviors in many insect species, sensitive, precise, and robust sensory systems are required (80. 81). Hostachy et al. (38) demonstrated that three functions could be measured by the dose-response curves of PER assay (the capacity to detect sugar, the capacity of sugar perception to initiate a PER, and a motor response to sugar if a PER is triggered).

In the current study, the score of PER of females *S. littoralis* activated after antennal stimulation increased with increasing sucrose concentration. Additionally, PER was recorded while the antennae were stimulated with water. These findings

suggested the presence of sugar and water-receptive neurons on the antennal gustatory sensilla of the female *S. littoralis*. The PER is positively associated with the activity of the antennal sugar GSNs and increases the rate of proboscis reflexes, which elicits food acceptance. The PER first reflects the integration of the gustatory perception and motivation for sugar and then allows feeding. This conclusion is also compatible with the current electrophysiological results from GSNs of both Vch and Lch, which showed greater responses to higher sucrose concentrations. The stronger response to the higher concentration of sucrose could trigger a stronger acquisition of sucrose at later processing stages in the brain. Hostachy et al. (38) argued that the dose responses of PER for sucrose in *Agrotis ipsilon* moth demonstrated the perception of sugar and the motor capabilities of sugar to elicit a PER. Sucrose-elicited PER has been described and implicated in associative learning in insects such as moths (38-41.82), butterflies (42), and bees (83). Understanding how sugar elicited PER works could aid in distinguishing between perception, motivation, and PER release pathways (38). Liscia and Solari (84), on the other hand, demonstrated that 0.1mM amiloride generated an electrical response from the deterrent neuron but had no effect on the behavioral activity of sucrose consumption in the blowfly. A significant negative association has been discovered in *H. virescens* larvae between the firing rate of the deterrent sinigrin-responsive GSNs and the amount of food ingested (85). Female moths forage more for food in the wild due to physiological and ecological needs to improve egg production and lifespan (75). Female insects use GSNs found in the gustatory sensilla on various regions of their body to select food or oviposition sites (4. 12. 86).

Conclusions

How insects interact with their environment and how neurons work at various stages of the sensory pathway are fundamental questions in the Neuroecology of herbivorous insects. The GSNs of antennal Vch and Lch gustatory sensilla have unique response characteristics to single ligands as well as compound mixtures. The various placements of both types of sensilla at the same antennal flagellomere showed divergent response profiles. The majority of sensilla showed multineuronal responses, showing that gustatory coding on the antenna of the female *S. littoralis* is composed of multiple channels. The PER is positively associated with the activity of the antennal sugar GSNs which elicits food acceptance. Understanding the molecular basis of polyphagy may provide opportunities for the development of new environmentally friendly pest control strategies, like push and pull. Tastants can be used to make pests consume more insecticide or less of other things, like crops.

List of abbreviations

GSNs: gustatory sensory neurons
s. chaetica: sensilla s. chaetica
N1: Neuron 1
N2: Neuron 2
N3: Neuron 3
N4: Neuron 4
Vch: ventral sensilla chaetica
Lch: lateral sensilla chaetica
R.H: relative humidity
LD: Light: Dark
PER: proboscis extension reflex
imp/s: impulses/ second.
mM: millimolar
mg: milligram
mL: milliliter
GRs: gustatory receptors

Declarations

Ethics approval and consent to participate.

Not applicable.

Availability of data and materials

All the data generated or analyzed during this study are included in this article.

Competing interests

The author declares that there are no competing interests.

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Author contributions

The author did the study conception and design. Material preparation, data collection, and analysis were performed by Mervat A. Seada. The first draft of the manuscript was written, read, and approved for the final manuscript by Mervat A. Seada.

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